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Centrosome Clustering in the Development of Bovine Binucleate Trophoblast Giant Cells

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Keywords
Placenta · Trophoblast · Polyploidy · Centrioles · Trophoblast giant cells

Abstract
Binucleate trophoblast giant cells (BNC) are the characteristic feature of the ruminant placenta. During their development, BNC pass through 2 acytokinetic mitoses and become binucleate with 2 tetraploid nuclei. In this study, we investigate the number and location of centrosomes in bovine BNC. Centrosomes typically consist of 2 centrioles surrounded by electron-dense pericentriolar material. Duplication of centrosomes is tightly linked to the cell cycle, which ensures that the number of centrosomes remains constant in proliferating diploid cells. Alterations of the cell cycle, which affect the number of chromosome sets, also affect the number of centrosomes. In this study, we use placentomal tissue from pregnant cows (gestational days 80–230) for immunohistochemical staining of γ-tubulin ($n = 3$) and transmission electron microscopy ($n = 3$). We show that mature BNC have 4 centrosomes with 8 centrioles, clustered in the angle between the 2 cell nuclei. During the second acytokinetic mitosis, the centrosomes must be clustered to form the poles of a bipolar spindle. In rare cases, centrosome clustering fails and tripolar mitosis leads to the formation of trinucleate "BNC". Generally, centrosome clustering occurs in polyploid tumor cells, which have an increased number of centrioles, but it is absent in proliferating diploid cells. Thus, inhibition of centrosome clustering in tumor cells is a novel promising strategy for cancer treatment. BNC are a cell population in which centrosome clustering occurs as part of the normal life history. Thus, they might be a good model for the study of the molecular mechanisms of centrosome clustering.

Abbreviations used in this paper

BNC	binucleate trophoblast giant cells
FC	fetal capillaries
FS	fetal stroma
G	gap
ME	maternal epithelium
S	synthesis
TE	trophoblast epithelium
TEM	transmission electron microscopy
TGC	trophoblast giant cells
TNC	trinucleate feto-maternal hybrid cell

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Introduction

In many mammalian species, trophoblast giant cells (TGC) undergo genome multiplication [Hoffman and Wooding, 1993; Zybina and Zybina, 2005]. In these TGC mitosis is reduced to a variable degree. In rodents, TGC perform repeated cycles of synthesis (S) and gap (G) phases without intervening mitoses. This leads to an enormous increase in DNA content – rat and mouse TGC reach DNA contents of about 500–2,000 C, respectively, where 1 C is the DNA content of a haploid cell. In TGC of artiodactyls, mitosis is reduced to a lesser degree. In camelid TGC, repeated incomplete mitoses lead to large lobulated nuclei [Klisch et al., 2005]. In the development of ruminant TGC, mitosis is usually complete, but cytokinesis is absent. These acytokinetic mitoses lead to the formation of binucleate TGC, i.e., binucleate trophoblast giant cells (BNC) [Wooding, 1992]. In cattle, these BNC undergo 2 subsequent acytokinetic mitoses, which result in BNC with 2 tetraploid nuclei [Klisch et al., 1999a; Klisch et al., 2004].

The centrosome in mammalian cells is a very dynamic structure with a central role in the organization of microtubules during interphase and mitosis. It consists of 2 centrioles surrounded by electron-dense pericentriolar material [Bettencourt-Dias et al., 2011; Conduit et al., 2015]. During interphase, the centrosome acts as the microtubule-organizing center of the cell and during mitosis the duplicated centrosomes form the 2 poles of the mitotic spindle. One pivotal centrosomal molecule is γ-tubulin, which is important for the nucleation of microtubules at the centrosome but also at spindle microtubules during mitosis [Watanabe et al., 2016].

Duplication of the centrosome is tightly linked to the cell cycle to ensure a constant centrosome number [Firat-Karalar and Stearns, 2014]. Consequently, alterations of the cell cycle lead to altered numbers of centrosomes. In polyploid tumor cells, an increased centrosome number can result in multipolar mitoses, which promote genome instability [Godinho and Pellman, 2014].

Since TGC typically show genome multiplication with altered cell cycles, aberrant centrosome numbers could be expected in these cells. In ruminant BNC, elevated centrosome numbers could be the possible cause of the occasional occurrence of tripolar mitoses and of trinucleate "BNC" [Klisch et al., 1999b]. Hence, we investigated the number of centrosomes in the development of bovine BNC.

Materials and Methods

Immunohistochemistry

Placentomal tissue from 3 pregnant cows (estimated fetal ages: 80, 95, and 210 days) was obtained from a slaughterhouse. Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 24 h and embedded in paraffin. Sections (2–3 μm) were dewaxed in xylol and rehydrated in graded ethanol. For antigen retrieval, the slides were heated in a microwave oven (600 W) in 0.01 M citrate buffer, pH 6.0, 3 times for 5 min. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 in methanol (30 min). Sections were blocked with 10% horse serum (Vector Laboratories, Inc.) and incubated overnight at 4°C with a monoclonal antibody against γ-tubulin (clone GTU-88, dilution 1:5,000) from Sigma-

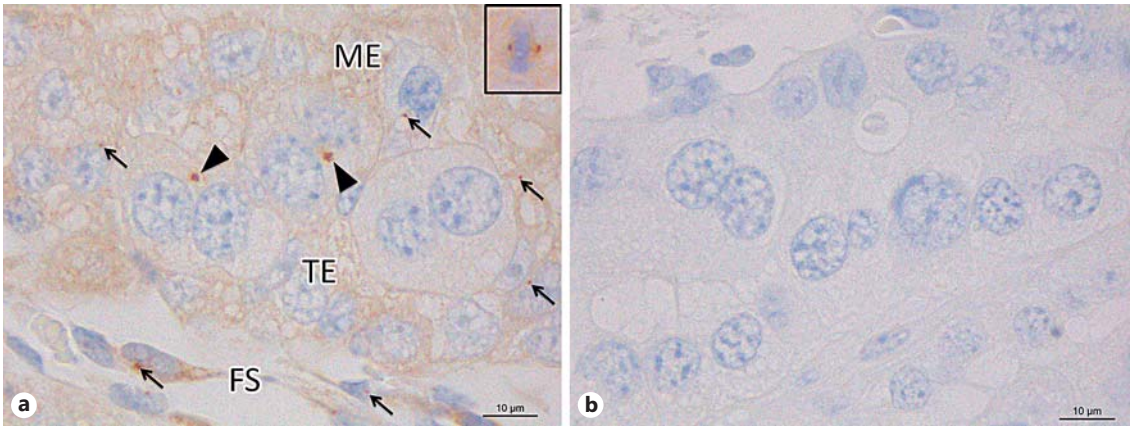

Fig. 1. a Immunohistochemical staining (brown) of γ-tubulin. In BNC, signals (arrowheads) are located in the angle between the 2 nuclei. The signal in BNC appears generally stronger than in other cell types (arrows). The **inset** shows a metaphase in which the spindle poles are immunolabeled. ME, maternal epithelium; TE, trophoblast epithelium; FS, fetal stroma. **b** Negative control in which the primary antibody was replaced by nonspecific IgG. Fetal age: 95 days.

Aldrich (St. Gallen, Switzerland). Control sections were incubated with nonspecific mouse IgG at a similar concentration. After washing, the sections were incubated with a biotinylated horse anti-mouse IgG (1:100; Vector Laboratories, Inc.) for 30 min and the signal was amplified with a streptavidin-peroxidase Vectastain ABC Kit (Vector Laboratories, Inc.). Antibodies were visualized using a Liquid DAB+ Substrate Kit (Dako Schweiz AG, Baar, Switzerland). Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Medite, Burgdorf, Germany).

Transmission Electron Microscopy

Placentomal tissue from 3 pregnant cows (estimated fetal ages: 120, 150, and 230 days) was fixed by perfusion through fetal blood vessels with 2.5% glutaraldehyde in 0.1 M Na/K phosphate buffer,

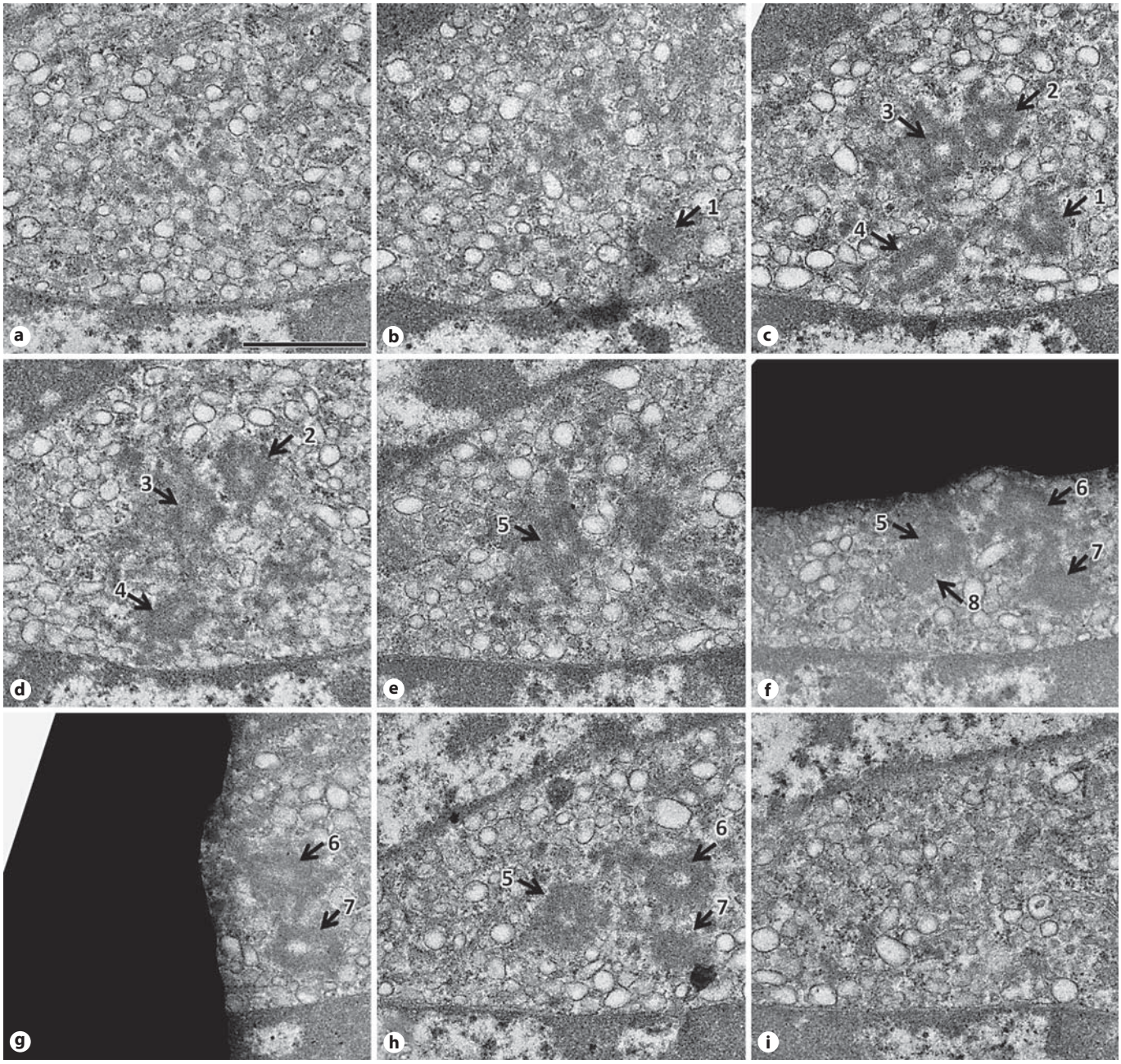

Fig. 2. Serial TEM sections of centrosomes in a BNC. One nucleus is visible in the lower part of the figures and the second nucleus appears in the upper left corner and increases through the series. The individual centrioles are numbered 1–8. Fetal age: 150 days. Scale bar, 1 μm.

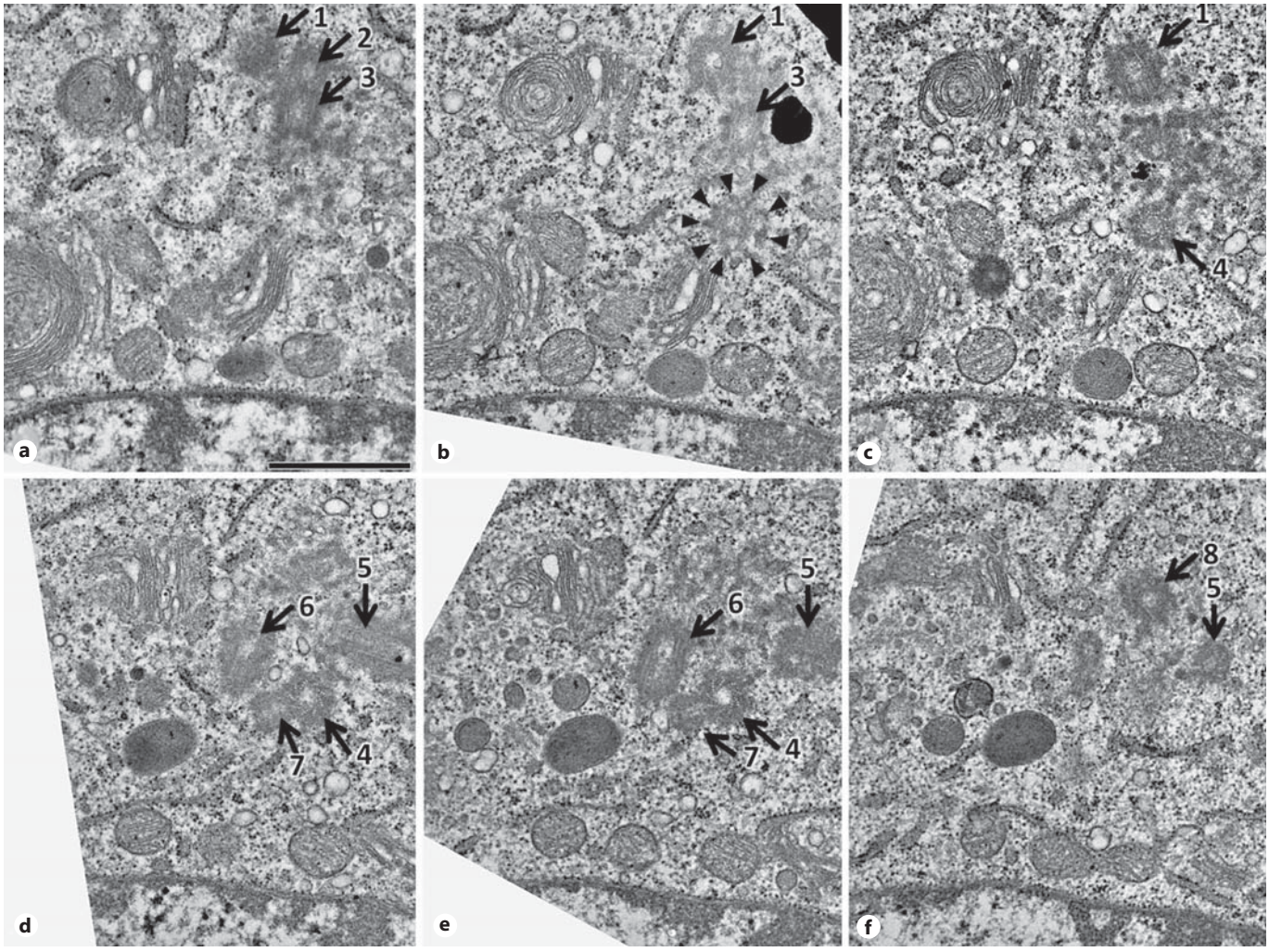

Fig. 3. Serial TEM sections of centrosomes. The centrioles are numbered 1–8. In **b** the distal appendages of centriole 4 are marked by arrowheads. The assignment of the centrioles into pairs is not always obvious, but pairs of centrioles seem to be arranged in approximately orthogonal orientation. These are: 1–6, 2–3, 4–7, and 5–8. Fetal age: 120 days. Scale bar, 1 μm.

pH 7.4, for about 5 min. After trimming the tissues into cubes of approximately 1 mm width, fixation was continued by immersion in the same fixative for 2 h at 4°C. Then the tissues were washed with 0.1 M Na/K phosphate, pH 7.4, overnight at 4°C and postfixed with 1% osmium tetroxide in 0.1 M Na/K phosphate for 1 h at 4°C, dehydrated in an ascending ethanol series starting at 70%, and, after transferring into acetone, embedded in Epon (Sigma-Aldrich, Buchs, Switzerland) at 4°C, followed by polymerization at 60°C for 2.5 days. Semithin sections, stained with 1% toluidinblue in 1% borax, were cut to select well-fixed areas of the tissue for ultrathin sectioning. Ultrathin sections of 60- to 80-nm thickness were cut and series of 12–20 sections were mounted on either 300/75 mesh grids or 1 × 2 mm slot grids, the latter with carbon-coated formvar support films. Sections were stained with uranyl acetate and lead citrate and analyzed using a transmission electron microscope (CM12; FEI, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000; Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV. For the analysis, BNC from sections which were central in a stack were scanned for the presence of centrioles. If centrioles were found, the same cell in adjacent sections of the stack was photographed. Due to the low number of observations, no statistics could be applied.

Results

Immunohistochemistry

Immunostaining of γ-tubulin produced a weak cytoplasmic background (Fig. 1a) which was slightly more intense in uterine epithelial cells than in trophoblast cells. Strong spots of immunostaining were seen close to the nuclei in a few cells. One spot or one dense cluster of spots

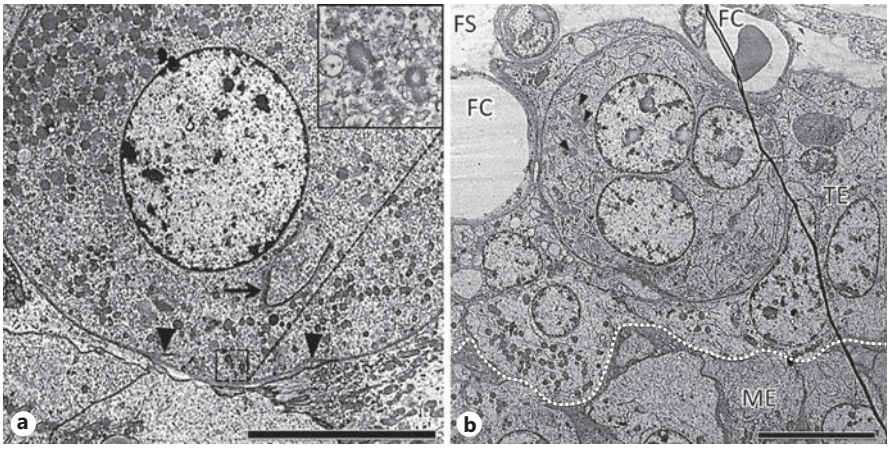

Fig. 4. a Mature migrating BNC with a pseudopodium; only 1 nucleus can be seen in this orientation. The pseudopodium is flanked by bundles of actin filaments (arrowheads) of the cortical cytoskeleton. Such bundles of actin detach from the cell membrane at the junction between the cell body and the pseudopodium. Two centrioles can be seen inside the pseudopodium (highlighted by the dotted square). These centrioles are shown at a higher magnification in the **inset**. The Golgi apparatus (arrow) is located close to the nucleus, at some distance from the centrioles. Fetal age: 120 days. Scale bar, 10 μm. **b** Trinucleate "BNC" located in the trophoblast epithelium (TE). Granules in the cytoplasm are indicated by arrowheads. In the fetal stroma (FS) 2 fetal capillaries (FC) are labeled. The feto-maternal interface, which separates the trophoblast from the maternal caruncular epithelium (ME), is marked by a dotted line. Fetal age: 150 days. Scale bar, 10 μm

per cell in interphase could be observed. In BNC this spot was generally larger than in other cell types and it was located in the angle between the 2 nuclei. In most BNC the signal was located in that part of the cell, which was directed towards the apical surface of the trophoblast epithelium. In mitotic cells (inset in Fig. 1a), the 2 spindle poles were labelled. No immunostaining could be seen in the negative controls (Fig. 1b).

Transmission Electron Microscopy

Centrosomes in BNC were usually found in the angle between the 2 cell nuclei (Fig. 2) in close association with the Golgi apparatus (Fig. 3). In serial sections, up to 8 centrioles could be identified, but the allocation into pairs was not always obvious. Two of these series of sections are shown (Fig. 2, 3), but further BNC with clusters of centrosomes with more than 4 centrioles were also found. Due to missing sections, folds, or limitations by the number of serial sections, these were not complete.

A different location of centrioles was found in one mature migrating BNC (Fig. 4a). In this cell, the centrioles were located at the base of a forming pseudopodium. In that position, the centrioles were at a greater distance from the Golgi apparatus than in most other BNC. The pseudopodium of BNC is a structure which is involved in the migration and fusion process of the cells [Wooding et al., 1994].

One "BNC" with 3 nuclear profiles is shown in Figure 4b. The vast majority of BNC had 2 nuclei. Due to sectioning, many of them showed only one nuclear profile in single sections. The "BNC" in Figure 4b shows the typical features of a mature BNC.

Discussion

In this study, we show that mature bovine BNC regularly contain up to 4 clustered centrosomes, with a total of 8 centrioles. Both γ-tubulin staining and transmission electron microscopy (TEM) revealed that these centrosomes are typically clustered in the angle between the 2 nuclei. γ-Tubulin is the essential molecule for microtubule nucleation at the microtubule-organizing center of the cell [Kollman et al., 2011], which is typically the centrosome. The presence of more than one centrosome in BNC was not an unexpected finding. BNC become binuclear by acytokinetic mitoses (Fig. 5) [Wooding, 1992; Klisch et al., 1999a] and consequently the 2 centrosomes, which form the mitotic spindle poles, remain in one cell. These 2 centrosomes with 4 centrioles are the expected finding in a binucleate cell with 2 diploid nuclei in the G_1 phase of the cell cycle. One ultrastructural image of a bovine BNC with 4 centrioles was shown by Björkmann [1970]. In our study we show that BNC regularly contain

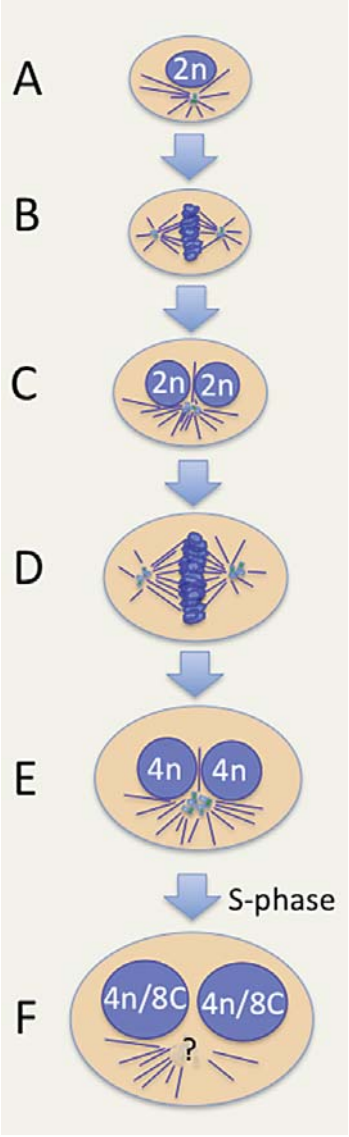

Fig. 5. Schematic drawing of stages of BNC development [modified from Klisch et al., 1999a]. Precursor cell of a BNC with 1 diploid nucleus and a single centrosome (A). First acytokinetic mitosis of the cell (B). The outcome of this first mitosis is a binucleate cell with 2 centrosomes (4 centrioles) (C). During the second acytokinetic mitosis, the centrosomes must be clustered to permit the formation of a bipolar spindle (D). After the second acytokinetic mitosis, the 2 nuclei each contain a tetraploid set of chromosomes. The 4 centrosomes (8 centrioles) are clustered in a juxtanuclear location (E). Previous studies [Klisch et al., 1999a, 2004] have shown that BNC pass through a further S phase, which leads to cells with 2 nuclei (F). Each of these nuclei contains a tetraploid set of chromosomes, but the DNA content is 8 C. In such cells 16 centrioles should be expected, but that was not observed in the present study.

up to 8 centrioles clustered together in a juxtanuclear location.

The number of observations was relatively small due to the technical limitations of serial sectioning. Since centrosomes are small structures of less than 1 μm in diameter and BNC are large cells of more than 20 μm in diameter, many BNC had to be examined to find the centrosomes. In this study we examined only a very limited number of placentas (3 for TEM and 3 for immunohistochemistry). Since the development of BNC seems to be a process that does not change greatly during pregnancy [Wooding, 1983, 1992; Klisch et al., 1999a], it appears justified to draw conclusions from this.

The presence of 8 centrioles in mature bovine BNC is in accordance with the fact that BNC become polyploid during their development [Klisch et al., 1999a]. Eight centrioles could be expected in the G_1 phase of a binucleate cell with 2 tetraploid nuclei. In previous studies it has been shown that bovine BNC with 2 tetraploid nuclei often pass through a further S phase, reaching a DNA content of 2 × 8 C [Klisch et al., 1999a, 2004]. In such cells 16 centrioles should be expected. We did not detect any BNC with this high number of centrioles. Possible causes of this could be that not all BNC reach this high DNA content and that we missed the few cells with 16 centrioles, or that the last round of DNA replication in the BNC is not linked to doubling of the centrioles.

During their development, BNC pass through 2 subsequent acytokinetic mitoses (Fig. 5) [Klisch et al., 1999a]. In the first mitosis the cell should have 2 centrosomes, one in each of the spindle poles. Due to the absence of cytokinesis, both centrosomes remain in the one cell. These 2 centrosomes are duplicated before the cell enters the second acytokinetic mitosis. In this second acytokinetic mitosis, all centrosomes remain in one cell again and this leads to BNC with 4 centrosomes. Such cells are shown in this study (Fig. 2, 3). In this second acytokinetic mitosis the centrosomes must be clustered into 2 poles of a bipolar spindle. This process of polyploidization via acytokinetic mitoses shows several similarities with polyploidization of hepatocytes [Guidotti et al., 2003]. One difference between BNC and hepatocyte polyploidization is that, in the latter, alternation of acytokinetic mitoses and complete mitoses with cytokinesis produces both binucleate and uninucleate polyploid cells.

Clustering of the centrosomes during the second acytokinetic mitosis of BNC is the precondition for a bipolar spindle and a binucleate cell is the outcome of that mitosis. Recently this process of centrosome clustering has gained considerable interest as a novel target for antitu-

mor therapies [Ogden et al., 2012]. In normal diploid cells, the single centrosome duplicates before mitosis and during mitosis the 2 centrosomes form the 2 spindle poles [Firat-Karalar and Stearns, 2014]. Thus, centrosome clustering does not occur in mitoses of diploid cells. For dividing polyploid cells, which are typically tumor cells, an elevated number of centrosomes creates a major problem. The supernumerous centrosomes can form multiple spindles poles. Such multipolar mitoses would in most cases lead to highly aneuploidy nonviable daughter cells [Ogden et al., 2012; McGee, 2015]. To avoid this, most polyploid cancer cells cluster the amplified centrosomes together, so that the cells have a "pseudo-bipolar" spindle during mitosis. Substances that interfere with centrosome clustering in tumor cells could induce multipolar mitoses with nonviable daughter cells and are thus potential chemotherapeutic agents.

The vast majority of BNC are truly binucleate, but rarely mononucleate and trinucleate "BNC" occur [Klisch et al., 1999b]. Such mononucleate or trinucleate "BNC" only differ from binucleate BNC in the number of nuclei, appearing to be similar in their location, shape, and cellular content. In Figure 4b we show a trinucleate "BNC", which is clearly not a trinucleate feto-maternal hybrid cell (TNC). True TNC result from a fusion of BNC with uninucleate maternal uterine epithelial cells and are located in the uterine epithelium. They condense, exocytose their granules, and undergo apoptosis and the remnants of the TNC are phagocytosed by uninucleate trophoblast cells [Wooding, 1992]. Trinucleate "BNC" are the outcome of rare tripolar mitoses [Klisch et al., 1999b]; these are relatively rare events and are primarily found in tumor cells [Kalatova et al., 2015]. It appears that in these cases centrosome clustering during mitosis was not successful. It would be interesting to see whether there are individual differences in the frequencies of tripolar mitoses in BNC development. Higher frequencies of tripolar mitosis should indicate a less efficient centrosome clustering due to genetic or environmental differences. Such cases could possibly be used to gain a deeper insight into the molecular and genetic mechanisms of centrosome clustering.

In the BNC, the centrosomes are clustered not only during mitosis but also during interphase. In tumors, such centrosome clustering throughout the cell cycle could be important for efficient cell migration [Ogden et al., 2013]. BNC are also migratory cells. They migrate through the apical tight junctions of the surrounding uninucleate trophoblast cells and fuse with a maternal uterine epithelial cell [Wooding, 1992; Wooding et al., 1994]. The position of the centrosome cluster in BNC is typically in that part of the cell directed towards the maternal epithelium. Growth of a pseudopodium by vesicle insertion is part of this process [Wooding et al., 1994] and it is very likely that microtubules are involved in the directed transport of vesicles into the pseudopodium. In Figure 4a we show centrioles in the pseudopodium of a mature granulated BNC, but it remains to be elucidated which role these and the associated microtubules might have during the process of BNC migration and fusion.

In summary, we showed that bovine BNC have an increased number of centrosomes. During interphase, these centrosomes are typically clustered in the angle between the 2 nuclei. Centrosome clustering, both in interphase and in mitosis, is typically found in tumor cells and inhibition of centrosome clustering is a promising strategy for anticancer therapies. BNC are one exceptional example of a nontumor cell population in which centrosome clustering is part of the normal life history of the cell.

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Conflicts of Interest

The authors declare no conflicts of interest.

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